CANCER

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Dr. Andervont
Dr. Gardner
Dr. Meier

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

NOV 1 2 1973

Application For Research Grant

Date:

1. Name of Investigator(s): (include Title and Degrees) Robert L. Herrmann, Ph.D.
Associate Professor of Biochemistry

2. Institution & Address: Boston University School of Medicine
80 E. Concord Street, Boston, Mass. 02118

3. Short Title of Project: Effect of Carcinogens On Nuclear Phosphoproteins and Phosphorylating Enzymes

4. Proposed Starting Date: March 1974

5. Anticipated Duration of this Specific Study: 3 years.

6. Brief Descripton of Objectives or Specific Aims:

We propose to examine the effects of various carcinogens on nuclear protein phosphorylation.

The steps in this process are planned as follows:

a.) Isolation and characterization of enzyme systems present in C57BL/6 mouse liver and lung nuclei which phosphorylate nuclear proteins.

b.) Examination of the effects of various carcinogens on these isolated enzyme systems.

c.) Comparison with the corresponding enzymatic activities isolated from normal, carcinogen-treated and tumor-bearing mice.

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7. Give a Brief Statement of your Working Hypothesis: Recent research has pointed out the

(Cont. on next page)

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important role of nuclear protein phosphorylation in the regulation of cell division and differentiation. In many animal and cell culture systems there is a co-relation between the functional state of a cell and its patterns of histone and non-histone chromosomal protein (NHCP) phosphorylation. Normally vs. abnormally proliferating cells and differentiated vs. embryonic or dededifferentiated cell types display characteristic differences in this regard. We propose to examine the effects of various carcinogens on nuclear protein phosphorylation with the expectation that these agents may significantly alter the enzyme systems involved. Such alterations may possibly be closely correlated with the establishment of the neoplastic state.

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

Our interest in nuclear protein phosphorylation stems from two types of observations, both of which bear on the cancer problem.

1) Histones and NHCPs each display transient, tissue- and growth-specific patterns of phosphorylation which appear to relate to transcriptive and replicative capability in the case of histones and to degree and quality of gene activation in the case of non-histones.

2) Many virus particles possess phosphorylated protein moieties which may play similar roles in cellular, as well as viral replication; and many of these viruses possess endogenous protein kinase activity capable of phosphorylating host cell as well as virion proteins in vitro.

Many published studies substantiate the relationship between chromosomal protein phosphorylation and the rate of cell division. Balhorn reports that in developing rat liver the level of F1 histone phosphorylation decreases from a high of 80% to undetectable levels between 15-day embryonic and adult animals, when the mitotic index has fallen to a few per cent of maximum. (Balhorn et al 1972a) (Cont. on next page.)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

See Page 23.

10. Additional Requirements: none

Biographical sketches of all principal and professional personnel (append)

See Page 24.

12. List of publications: (Five most recent as pertinent) (append)

See Page 25.

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13. Budget: (1st year)

	% time	Amount
A. Salaries (Personnel by names)		
Professional Robert L. Herrmann, Principal Investigator	15	REDACTED
Technical Technical Assistant, to be selected	50	REDACTED
Fringe benefits		REDACTED
Sub-Total		
B. Consumable Supplies (list by categories)		
Radioisotopes		1,000
Enzymes, Reagents		500
Drugs		300
Glassware		800
Sub-Total		2,600
C. Other Expenses (itemize)		
Animals - C57BL/6 &BALBc mice		500
Animal Care		400
Sub-Total		900
D. Permanent Equipment (itemize)		
Incubator-Shaker - Precision		850
Replacement Rotor		400
Servall SS34 for RC2B centrifuge		
Fraction Collector Buchler fractomette		1,000
		2,250
		1,185
E. Overhead (15% of A+B+C)		
Total		11,335

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	R	2,000	1,200	1,000	1,230	10,430
Year 3	R	2,200	1,500	800	1,380	11,380

Signature Robert L. Herrmann (617) 262-4200 x6415
Director of Project
Telephone

Signature Robert N. Jacobs 617 262-4200 x6101
Business Officer of the Institution
Telephone

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

	Title of Project	Source	Amount	Duration
Current				
	Genetic Recombination in E. coli	National Science Foundation	$38,000	2 yrs. 12/15/72-12/14/74
Pending				
	Transcriptional Diversity in Aging Human Brain	U.S.P.H.S.	92,984	3 yrs. 1/1/74-12/31/76
	*Nuclear Phosphoproteins and Phosphorylating Enzymes in Neoplasia	U.S.P.H.S.	69,790	3 yrs. 1/1/74-12/31/76
	** Effect of Carcinogens on Nuclear Phosphoproteins and Phosphorylating Enzymes	Damon Runyon Cancer Fund	19,730	3/1/74-2/28/75

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* We have applied to the U. S. Public Health Service for funds to isolate and characterize histone and non-histone proteins from chromatin of human colon and colon carcinoma and from 3T3 cells and their SV-40-transformed counterparts. Some of the purification work outlined in that proposal will follow similar procedures to those proposed here, but there are so many differences involved in using mouse tissues and the in vivo studies with the mouse that we regard the two proposals as essentially distinct.

** The application to the Damon Runyon Fund is essentially a duplicate of the present proposal. However, the breadth of the proposal is so great that we would like to request consideration of at least partial support even if the Damon Runyon grant is funded. This would allow us an earlier start on both invitro and in vivo phases of the investigation and also to place greater emphasis on the tobacco smoke condemsate studies.

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8. Cont. from page 2.
This extensive modification of fine structure of F1 histone is also observed in regenerating liver. (Balhorn et al, 1971) Considerable microheterogeneity due to phosphorylation of F1 is seen in rapidly dividing Morris rat hepatomas, mouse mammary carcinoma, Lewis mouse lung tumor, mouse skin melanoma, and Shear mouse glioma in log phase culture growth versus stationary phase. (Balhorn et al, 1972b). There is also a linear correlation between growth rate and F1 phosphorylation in the Morris series of minimal deviation hepatomas.

Others have shown (Gurley et al, 1973) that massive F1 and F3 phosphorylation occurs during the G2-M transition in cultured Chinese hamster ovary cells.

Langan has demonstrated a more limited and specific phosphorylation of F1 which is stimulated in liver by glucagon or cyclic AMP administration (Langan, 1968). Two amino acid sites - ser 37 and ser 106 - have been identified as the phosphorylated residues, but only the ser 37 site is modified by a cAMP- (and presumably glucagon-) dependent enzyme. This group has also demonstrated a 40-100% increase in template activity in vitro by reconstituting chromatin with this specifically phosphorylated F1 histone. (Watson and Langan, 1973) Others have shown an increase in ^{3}H-Actinomycin D binding, as well as increased template activity, in calf thymus chromatin preparations treated with pineal gland protein kinase (Fontana and Lovenberg, 1973).

Because of the foregoing evidence, it seems probable that histones, especially lysine-rich F1, play a structural role in the maintenance and regulation of chromatin and that hormonally-mediated or proliferation-associated phosphorylation allows access of polymerizing enzymes to naked DNA by 1) removal of the proteins or 2) relaxation of steric or configurational restraints on the chromatin (possibly supercoiling). This latter suggestion is supported by work (Adler et al, 1971) showing that in reconstituted F1-DNA complexes, phosphorylated F1 is less effective than the unmodified species in altering the DNA contribution to the circular dichroic spectrum of the complex, i.e. there is less interaction between phospho-F1 and DNA.

Evidence is also accumulating that levels and patterns of phosphorylation of other, acidic nuclear proteins (non-histone chromosomal proteins, NHCPs) are directly related to various parameters of differentiation or gene activation. Several groups (Shea and Kleinsmith, 1973; Kostraba and Wang, 1972; Chae et al, 1972; Martelo et al, 1970) have demonstrated tissue-specific patterns of NHCP phosphorylation as well as tissue-specific spectra of NHCPs. Allfrey (1973) proposes a role for NHCP phosphorylation in the regulation of transcription and cites as evidence several key observations: 1) phosphorylated NHCPs

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stimulate transcription in vitro from DNA of homologous tissue, 2) dephosphorylation destroys this stimulatory effect, 3) uptake of ^{32}P in vivo correlates with gene activation in PHA-stimulated lymphocytes and ecdysone-treated insect chromosomes, 4) in liver, cAMP and cortisol stimulate a selective phosphorylation of specific NHCPs, 5) cells with low levels of RNA synthesis, e.g. mature avian erythrocytes, display decreased levels of NHCP phosphorylation, and 6) in synchronized HeLa cell cultures, the lowest levels of NHCP phosphorylation correspond to the G2-M phase of the cell cycle when RNA synthesis is at a minimum.

Evidence for phosphorylation-mediated activation of a specific protein of known function is provided by the work of Martelo (1973) who reports that rabbit skeletal muscle protein kinase stimulates DNA-dependent RNA polymerase from E. coli (with T4 phage DNA as template) by selectively placing a phosphate on a single serine residue of the sigma factor.

Taken together, all of these observations strongly suggest that abberations in the control of nuclear protein phosphorylation may be crucial in the etiology of neoplastic growth. Histone phosphorylation, with its correlation to cell division rate, and NHCP modification, with its relation to gene activation, are thus important to a complete understanding of the control of cell function.

As in the past, a clear definition of the nature of metabolic processes has depended on characterization of the associated enzymatic activities. Therefore, we propose to initially carry out an investigation of the phosphorylating enzyme(s) (protein kinases or PKs) which catalyze these protein modifications in mammalian tissues. So far PKs which phosphorylate histone have been found in a wide variety of species and tissues. (Kuo and Greengard, 1969). cAMP-dependent kinases have been implicated in control of mammalian protein synthesis, (Walton et al, 1971) bacterial RNA synthesis, (Eron et al, 1971) and mammalian synaptic transmission, (Greengard, 1973) as well as in glycolysis (activation of phosphorylase b). As of this writing, the enzymes responsible for NHCP and histone phosphorylation are poorly characterized, although the following mechanism is generally accepted (Kumon et al, 1972; Gill and Garren 1971; Corbin et al, 1972) RC + cAMP $\rightleftharpoons$ R-cAMP + C, where R represents one or more regulatory subunits, C is the active catalytic subunit, and RC represents the inactive holoenzyme.

Several groups have reported the isolation of PKs from the nuclear compartment of various cell types; this finding is important in light of the importance of phosphorylated macromolecules in the nucleus. Reddon and Anderson (1972) have isolated four cAMP-

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independent PK activities from rat liver by phosphocellulose chromatography. Our laboratory has also resolved four enzymes, using DEAE chromatography (see below under Preliminary Work) It seems highly likely that there should be at least two distinct kinase activities in nuclei, since enzymes have been partially purified which prefer either histones (Lake and Salzman, 1972; Takeda et al, 1971; Siebert et al, 1971) or NHCPs (Martelo, 1973) but not both. The conflicting reports in the literature concerning cAMP-dependence of kinases isolated by various laboratories undoubtedly stems in part from transient or cell compartmental fluctuations in cAMP levels (i.e., adenylate cyclase or cyclic nucleotide phosphodiesterase activities) or artifactual concentration changes introduced because of dilution during preparation. These dilutions could affect the equilibrium reaction described above. Other enzymes whose presence may hamper interpretation of data are the ATPases and phosphatases which are present in most subcellular fractions, including nuclei. (A discussion of these problems and how they can be overcome is given in Corbin et al, 1973a,b)

The system we have chosen for study is the C57BL/6 mouse, an animal we have studied for several years for possible age-dependent changes in chromatin template activity (O'Meara and Herrmann, (1972)). Early experiments will require only normal animals; later experiments will require carcinogin-treated and tumor bearing animals. At that time we plan to treat a portion of our mouse colony with benzo(a) pyrene, tobacco smoke condensates, and urethane and to examine isolated chromatin proteins and protein kinases.

Because our mouse colony is presently being maintained as an aging colony, we plan also to examine the effect of age of both carcinogen-treated and untreated animals upon the levels of nuclear protein phosphorylation and protein kinase activity in liver and lung tissues.

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Preliminary experiments have been carried out with rat liver with the goal of developing a satisfactory method of extracting protein kinase activity from whole nuclei, prior to an investigation of kinetics, substrate specificity, and regulation. Nuclei prepared by the Blobel and Potter method from rat liver have been carried through the successive Tris washes described by Huang and Huang 1969 for solubilization of chromatin. This technique involves two rinses in .075 M NaCl-.025 M EDTA, pH 8, followed by two washes in each of the following Tris buffer solutions, also pH 8: .05 M, .01 M, .002 M, .0004 M, and finally distilled water. Each wash begins by dispersion of the preceding pellet with a Potter-Elvejehm homogenizer and ends with a 7000g centrifugation. When these extracts were assayed for kinase activity, it was found that 62% of the total nuclear PK activity was solubilized by the first three washes (two saline-EDTA and one .05 M Tris wash). This activity was stimulated three-fold by 5×10^{-6}M cAMP. The surprising finding that so much activity

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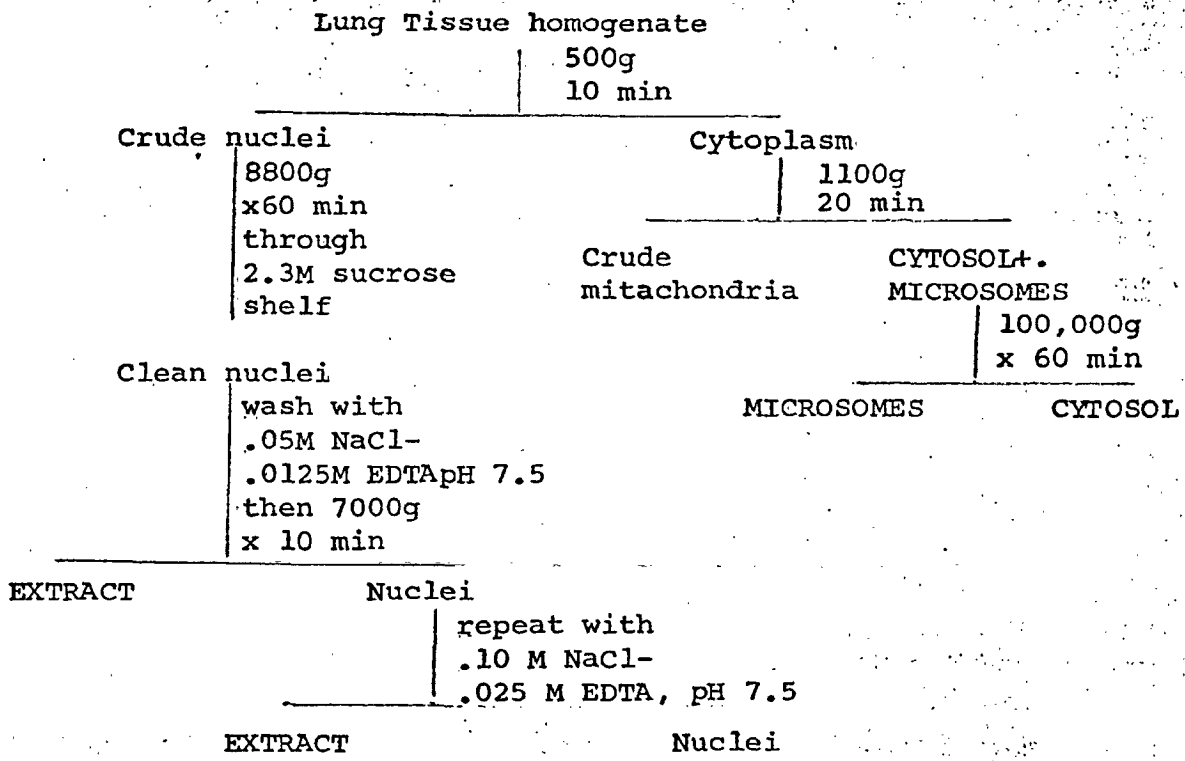
could be released by such mild treatment of nuclei prompted us to repeat this type of experiment with dog lung tissue which possesses a higher degree of connective tissue infiltration, to insure that the technique would serve for the proposed lung studies. Sub-cellular fractions were prepared according to the following scheme:



The nuclear extracts were pooled and the subcellular fractions designated by UPPER CASE letters were assayed for protein kinase activity in our standard assay (see Methods section for details). The data shown in Table I verifies that low salt washing is sufficient to extract considerable protein kinase activity from whole lung nuclei. Also shown is the fact that the protein solubilized is much richer in PK activity than any cytoplasmic fractions. Experiments with these pooled nuclear extracts have allowed us to determine approximate pH optimum (6.0) (see figure 1) and K_m for cAMP (5×10^{-7}M) (see figure 2). These values doubtless represent several histone-phosphorylating enzymes in dog lung cell nuclei and they will be re-evaluated as we fractionate and purify the enzymes. A similar study was also carried out with rabbit kidney cells to examine the effect of infection with herpes virus HSV-1. A significant difference between infected and non-infected cells was observed, as indicated in Figure 3.

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In an attempt to further resolve nuclear PK activities, we have chromatographed the .05 M and .10 M NaCl extracts this time from nuclei of rat liver, on DEAE-cellulose. Figure 4 below shows the apparent resolution of four PK activities clearly. It can be seen that the proteins extracted by .10 M NaCl are qualitatively similar to those in the .05 saline extract (the UV-absorbing material eluted at high ionic strength - 0.5 M KCl - probably represents nucleic acid precursors and low molecular weight RNA). Also to be noted is the marked enrichment of the PK (peak III in figure 3) eluting at 0.3 M KCl. This peak III is currently under investigation.

Table I. Protein kinase activity in subcellular fractions of dog lung.

Enzyme source + or - 5 uM cAMP	µg protein	pmoles ^{32}P transferred	$\frac{\text{pmoles } ^{32}P}{\mu g \text{ protein}}$	cAMP stimulation
Combined nuclear extracts (+)	9.2	25,900	2815	1.6-fold
Combined nuclear extracts (-)	9.2	16,100	1750	
Cytosol plus microsomes (+)	395	99,400	252	3.7-fold
Cytosol plus microsomes (-)	395	27,000	68	
Cytosol (+)	375	277,000	741	4.0-fold
Cytosol (-)	375	68,900	184	
Microsomes (+)	120	4,900	41	2.2-fold
Microsomes (-)	120	2,300	19	

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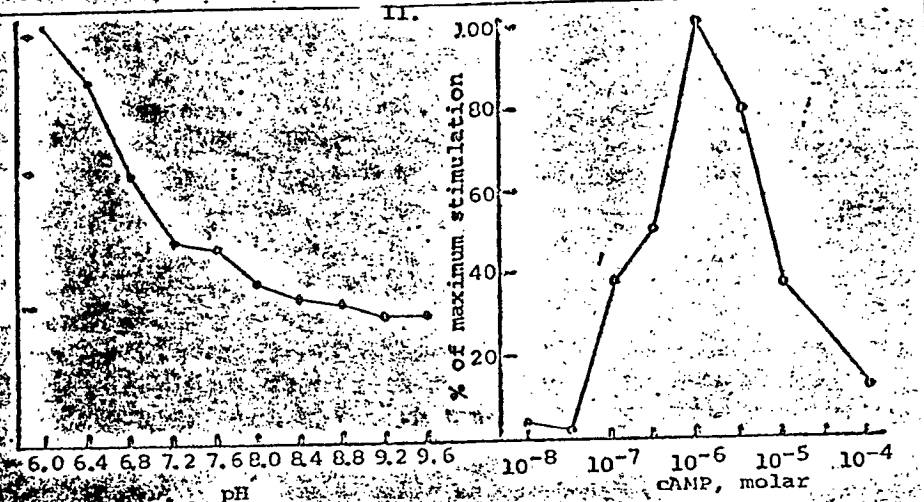

Figures 1 and 2. cAMP concentration and pH dependence of protein kinase activity in pooled .05 M and .10 M NaCl nuclear extracts from dog lung (standard assay as described in Methods). In fig.1 cAMP = 5×10^{-6}M; in fig.2 pH = 6.5.

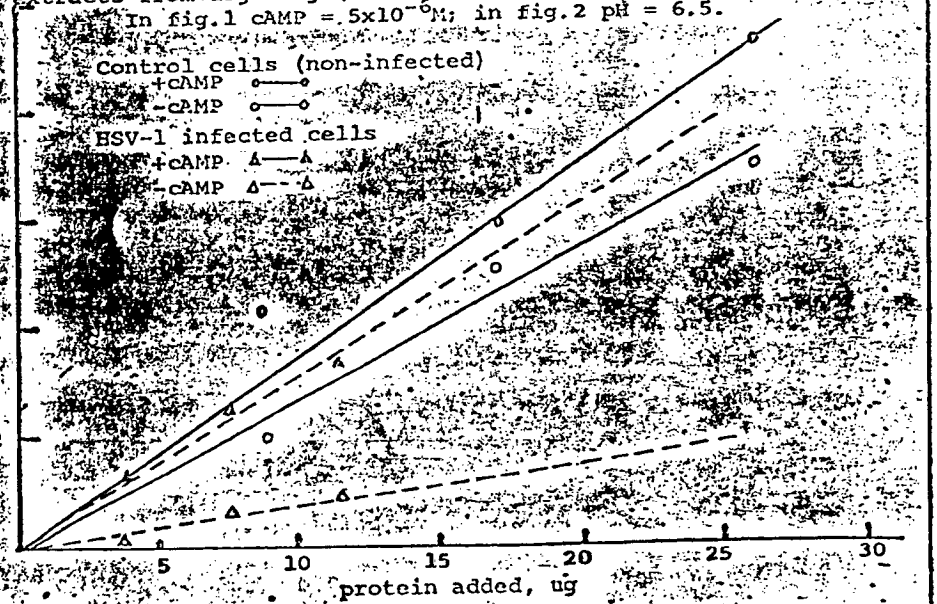

Figure 3. Log-phase rabbit kidney cells were harvested, sonicated, and assayed for PK activity in standard assay. Graph shows linear dependence of activity on added cell protein. When included, cAMP was 5×10^{-6}M.

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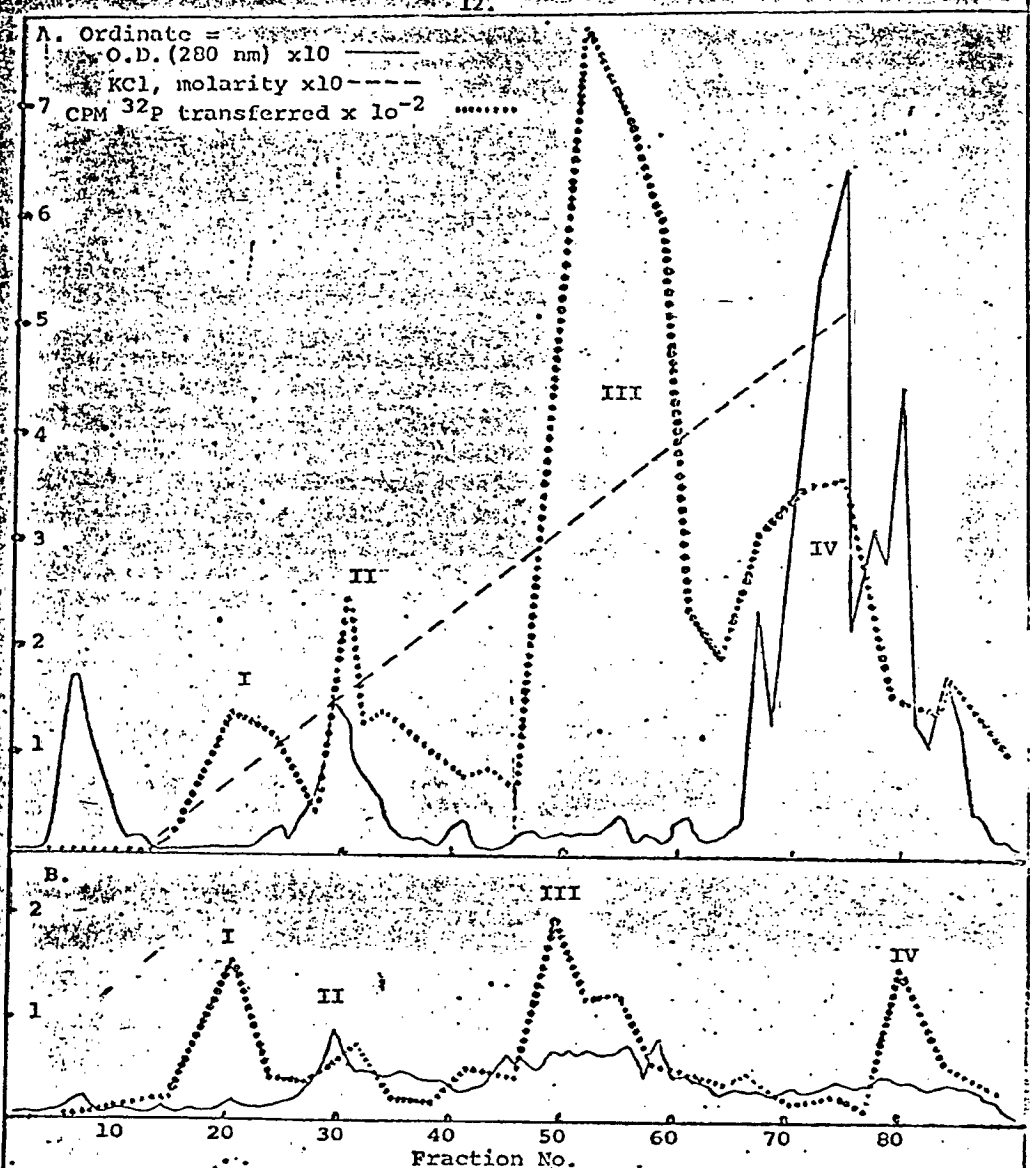

Figure 4. Approximately 3 mg of .05 M(Panel A) and .10 M(Panel B) NaCl nuclear extracts was applied to a 1 x 10 cm DEAE-cellulose column and eluted with a 100 ml gradient of 5-500 mM KCl, 10mM Tris, pH 7.4. Fractions were assayed for protein kinase activity(standard assay described in Methods) with cAMP included at 5×10^{-6}M.

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2. Experimental Procedures

a) Preparation of Tissues

C57BL/6 mice will be obtained from Jackson Laboratories, Bar Harbor, Maine. Animals will be maintained in the animal laboratory of the V.A. Hospital, Bedford, Mass. All other operations will be carried out at the Boston University Medical Center.

b) Nucleoprotein preparation and analysis

Cell Nuclei will be prepared in this laboratory by the method of Blobel and Potter (1966). Tissues are dissected as free as possible of connective tissue and, in the case of tumors, necrotic patches. This is followed by extensive washing in isotonic sucrose (0.25M). The tissue is finely minced, homogenized in a Dounce glass homogenizer with loose pestle, and filtered through cheesecloth. Crude nuclei and cytoplasm are prepared by a 10-minute, 500xg centrifugation. Clean nuclei are prepared by centrifugation of the crude nuclear pellet (after dispersion with the loose pestle in 1.7M sucrose) through a shelf of 2.3M sucrose. Nuclei prepared in this manner are relatively free of membranous cytoplasmic contaminants as verified by phase contrast microscopy. This method has proved quite adequate for our work with rodent tissues (rat and mouse) and for our preliminary studies with dog lung. We are currently exploring an alternate nuclear preparation method based on the work of Magliozzi et al., (1971), who have overcome the difficulty of working with mammalian tumor tissue by the inclusion of low concentrations of lead ($.002M\ PbAc_2$) and Triton N-101, a non-ionic detergent, in initial fractionation steps. It is claimed from electron microscopic evidence that this method yields absolutely clean nuclei while inhibiting nuclear autolysis and enzymatic degradation of chromosomal proteins (with a yield of 90%). Naturally, the effect of Pb^{++} on PK activity will be determined. Another alternative is the Chauveau method (1956) as it has been modified for tumor tissue by Siebert (1967) or Busch et al (1968).

Whole histones will be isolated from nuclei by the acid extraction technique of Panyim et al (1971). F1 histone will be extracted from nuclei, whole histones or chromatin by the Johns method (1964). The lysine-rich F1 fraction will be further purified and fractionated on Bio-Rex 70 columns eluted with a stepwise gradient of 8-40% guanadinium HCl. (Hohmann and Cole, 1971) Electrophoretic analysis of histones will be carried out on pH 2.3 acrylamide-urea acetic acid gels. Purified lysine-rich

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histones are resolved into F1 phosphorylated derivatives by 40hr. electrophoresis on 25cm gels. (Panyim and Chalkley, 1969) When desired, F1 histones are dephosphorylated by treatment with E. coli alkaline phosphatase. (Balhorn et al, 1971). β-mercaptoethanol and $NaHSO_3$ will be included in the histone preparative buffers to inhibit aggregation (primarily formation of F3 dimers) and histone protease activity, respectively.

A mild treatment for the isolation of NHCP's will be employed, involving extraction of purified nuclei with 1.0 M NaCl followed by DEAE-cellulose chromatography. (Wang, 1967; Kamiyama and Wang, 1971)

Since this method resolves significant quantities of phosphoproteins, alkali-labile phosphate content will be determined (Kleinsmith et al, 1966; Berenblum, 1938). NHCPs will be fractionated on SDS-Tris gels according to the method of Van den Broek et al (1973). There is a gentle method for isolating histones and NHCPs from the same tissue, which we are currently experimenting with. Chromatin is prepared from nuclei by successively decreasing ionic strength Tris buffer washes until the chromatin is finally solubilized in distilled water (Huang and Huang, 1969) It is then sheared in a Virtis homogenizer in 3.0M NaCl; protein is separated from DNA on a Bio-gel A-50m column. Histones are removed from the pooled protein peaks (after concentration) by chromatography on Bio-Res 70 and the NHCPs comprising the run-off from this column are fractionated on DEAE-cellulose. (Elgin and Bonner, 1972). All gels will be analyzed after staining for determination of protein bands using a Gilford spectrophotometer equipped with a gel-scanning attachment.

c) Protein kinase isolation, assay, and characterization

Our standard assay for protein kinase activity is a modification of the method of Greengard et al (1969). It contains, in a total volume of 0.2 ml : 15 mM potassium phosphate buffer, pH 6.5; 2.5mM NaF; 0.3 mM EGTA; 2.0 mM theophylline; 6.2×10^{-8}M ATP having 10^5-10^6 dpm of $AT^{32}P$; 500 µg of calf thymus whole histone; and enzyme preparation. When called for, cAMP is added at 5×10^{-6}M. Incubation time, except for time-course studies, is fifteen minutes at 37°C. The reaction is stopped by addition of 2 ml of ice cold 20% TCA-0.25% $NaWO_4$ containing 0.1 M Na_3PO_4. 0.63% bovine serum albumin is added (0.2 ml) as a carrier protein. The precipitate is removed by means of a ten minute, 2500xg centrifugation and resolubilized in 1.0M NaOH. This procedure is repeated twice and the final precipitate is counted in NaOH and Aquasol in a liquid scintillation spectrometer. For determination of enzymatic incorporation

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of phosphate into endogenous protein, the histone addition is omitted from the above procedure.

desired, F1 histones are dephosphorylated by treatment with E. coli

We have employed several methods of PK preparation. Total soluble PK can be prepared by the Greengard method. Nuclear PK activity has been prepared by a variety of techniques. (Takada et al, 1971; Lake and Salzman, 1972; Siebert et al, 1971) All involve salt extraction of nuclei. A further purification step involves chromatography on a phosphocellulose or DEAE cellulose column. We find that extraction of nuclei twice with .075M NaCl-.025 M EDTA pH 8, followed by two washes with 0.05 Tris pH 8 is sufficient to extract more than 60% of the kinase activity from preparations of rat liver nuclei. We plan to determine optimum salt concentration, pH of extraction, and cellulose chromatography conditions for the isolation of this soluble or weakly bound fraction of kinase activity, as well as the residual PK activity, which is either chromatin or nuclear membrane-bound. We will determine the feasibility of an early gel filtration or DEAE-Sephadex step to separate holoenzyme from activated catalytic subunit and cAMP bound regulatory subunit. We are also investigating the possibility of preparing an affinity column of purified F1 histone coupled to Sepharose 4B. (Cuatrecasas and Anfinsen, 1971) Enzyme preparations which have been purified through one or two DEAE steps will be passed through this affinity column and PK will be specifically eluted with excess F1 histone. Alternative purification procedures will include isoelectric focussing or sucrose gradient centrifugation. All enzyme preparations will be assayed for ATPase and phosphodiesterase activity by the method of Corbin et al (1972). Since pre-existing levels of activation prior to PK preparation are important in dictating the physical state of the holoenzyme (and since degree of activation of cAMP dependent PK is related to cAMP levels) all tissues will be assayed for cAMP with the assistance of Dr. Peter Polgar, of our Department of Microbiology, who has extensive experience in the measurement of cyclic nucleotide concentration and adenyl cyclase activity. (Polgar et al, 1973). Whole tissues, of course, will also be assayed for gross levels of PK activity and cyclic nucleotide response.

Purified PK activities will be electrophoresed (on SDS gels), centrifuged in sucrose gradients, and run on Sephadex columns to determine molecular weights. Kinetics of ^{32}P transfer will be examined and optimum temperature determined. Optimum metal ion concentrations and ionic strength will be investigated as well as the K_m for cAMP, ATP, and substrate.

In order to determine natural substrates for the kinase activities isolated, we will add whole histone or NHCP prepared

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from mouse tissues to the standard protein kinase assay in lieu of calf thymus histone. The final TCA precipitate will then be divided and electrophoresed on identical urea (for histones) or SDS-Tris (for NHCPs) gels. One gel will be stained and scanned for protein. The other will be sliced and counted for determination of the extent of ^{32}P incorporation into protein peaks. In addition, we will attempt to utilize the new method of two-dimensional gel electrophoresis (Orrick et al, 1973) of nuclear proteins to determine differences between control cells and cells from carcinogen-treated animals. This technique, which yields better resolution of NHCPs than ordinary one-dimensional electrophoresis, should allow us to determine cancer-specific phosphorylated nuclear acidic proteins. On the other hand, our current gel system for histones is quite adequate for investigation of these proteins. Alternatively, nucleoprotein fractionated by the methods described in the preceding section will be utilized as substrate in the standard assay. Protein will be determined in the TCA precipitate, normalized with respect to assay conditions and protein concentration, and in this manner relative substrate preference of the enzyme(s) will be ascertained.

d) Carcinogen Studies

Following the isolation and partial purification of the nuclear PK's we next propose to examine the effects of various carcinogens which have been implicated in the alteration of the structure and function of DNA and to compare their effects with those of tobacco smoke condensates. These known carcinogens include alkylating agents such as the nitrosamides and alkane sulfonates which have been shown to act directly at the DNA and RNA levels to bring about alkylation of the N7 position of guanine, the N1 and N3 position of adenine and the N1 position of cytosine. In addition, we will examine the ethylating reagent nitrosoethylurea which has also been shown to directly alkylate the N7 position of guanine. These studies have been reviewed by Magee and Barnes (1967). Inhibition studies will be carried out with levels of carcinogen which approximate the physiological concentrations effective in carcinogenesis. Comparison with tobacco smoke condensate will be carried out using the preparative technique of Sydnor, Allen and Higgins (1972).

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Although the nitrosamides and alkane sulfonates are believed, generally, to function directly, without the requirement of metabolic conversion to an active form, it is planned to examine their effects in the intact mouse with the expectation that there may well be significant effects which are secondary to their alkylating activities

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which may depend upon additional metabolic steps. Mice will be injected with N-Methyl-nitrosourea (≃100 mg/kg body weight) or methylmethane sulfonate (50 mg/kg body weight) and liver and lung tissue removed and treated for the isolation of nuclear phosphoproteins and PK's as described above.

Because the carcinogens of the N-nitrosamine group have been detected in tobacco smoke condensate (Rhoades and Johnson, 1972), we also plan to study the very potent carcinogen, dimethylnitrosoamine (20mg/kg body weight) which is known to require prior metabolic conversion to an active form. Here, BALB/c mice will be used as well as C57BL/6 mice, and newborn mice will be used as well as adult animals (Toth et al, 1964). Comparison with the effects of whole tobacco smoke condensate will be carried out with the water-soluble extract prepared as described by Sydnor et al (1972), administered in the dose range 0.25-1.0 mg/ml of 5% sucrose solution in the drinking water. Special care will be devoted to the possible presence of new or altered PK's in the carcinogen-treated animals; any such enzymes would be thoroughly examined as a possible target for a new chemotherapeutic approach.

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e. Potential Importance of this Work to the Cancer Problem

The etiology of cancer has been variously associated with cell alterations leading to cell proliferation or dedifferentiation. We have attempted to show the crucial position which nuclear protein phosphorylation appears to occupy in the regulation of these metabolic processes. Our proposal involves the characterization of the enzymes and substrates involved in the phosphorylation process, and includes a comparison both in vitro and in vivo of carcinogenic agents, which are known to function at the nuclear level, with tobacco smoke condensate. Our overall goal is to seek for new or altered nuclear phosphorylating enzymes which appear as the result of carcinogen treatment, to fully characterize these activities and to describe the mechanism of their production.

f. Previous work by applicant

Past studies from this laboratory which relate to this proposal follow two lines. We have been interested in the effects of the acridine dyes upon the replication of the episomal F factor of E. coli strain K-12. (Samaha et al 1967) and our most recent results have posed the interesting possibility that the acridines can function as inhibitors of DNA polymerase I in E. coli not only by binding to the DNA substrate but also by binding - apparently at the active site - to the enzyme. (Eberhard and Herrmann, 1972). Our second line of interest has covered possible age-dependent modifications in the structure and template activity of mouse liver chromatin (O'Meara and Herrmann, 1972). We therefore feel confident that we can carry out the enzyme isolation and kinetic studies outlined in this proposal, and we have had considerable experience with the preparation of mouse chromatin and its fractionation into histone and non-histone components.

We have also worked with the cyclic AMP-dependent differentiation of the mouse C1300 Neuroblastoma cell in tissue culture (Burstein, Neumann and Herrmann 1972) and this has given us some appreciation for the intricacies of cyclic AMP involvement in metabolic control processes.

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9. The proposed work would be carried out in the laboratory of the principal investigator in the Medical Research Building of the Boston University Medical Center. The laboratory occupies 1200 sq. ft. and includes a room for high voltage and disc gel electrophoresis and a large laboratory for enzymology and protein fractionation. There are also adjacent facilities shared with two other investigators; a large cold room, a hot room, and a preparation room. Equipment in the laboratory of the principal investigator includes a liquid scintillation spectrometer, Gilford spectrophotometer with linear transport for gel scanning, fraction collectors and an RC-2B centrifuge. Also available are a Technicon amino acid analyzer, Spinco L2-50 and Model E analytical ultra-centrifuges, and an RCA-EMU-3G electron microscope.

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Five Most Relevant Publications:

1. The Effect of Iododeoxyuridine upon Conjugation and the Fate of Transferred DNA in E. coli K-12. A.D. Cooper, M.W. Burgan, C.W. White and R.L. Herrmann, J. Bacteriol., 107 433 (1971).

2. Effect of DNA Ligands on Deoxyribonucleases and DNA Polymerase I of E. coli K-12. C. Eberhard and R.L. Herrmann J. Bacteriol. 112 224-230 (1972).

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5. Effect of Iododeoxyuridine on Cell Division in Recombination-deficient Mutants of E. coli K-12. D.M. Hanson & C.W. White and R.L. Herrmann. Abstracts, 73rd Ann. Mtg. Amer. Soc. Microbiol. Miami Beach 1973.

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